PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Mary R. FLACK et al.

Serial No.: 08/379,872 Group: 1205

Filed: January 27, 1995 Examiner: J. Goldberg

For: GOSSYPOL FOR THE TREATMENT OF CANCER

37 CFR § 1.132 DECLARATION

Honorable Commissioner of Patents
and Trademarks
Washington, DC 20231

Sir:

I, Richard Knazek, declare as follows.

1. I am a co-inventor of the subject matter that is disclosed and claimed in the matter of the above-identified application, and I am an expert in the field of the present invention as evidenced by the attached copy of my *curriculum vitae*.

2. I am also a co-author with Y-W. Wu and C. L. Chik of the scientific paper that is attached to the present Declaration [Wu et al., CANCER RESEARCH 49, 3754-3758, July 15, 1989], which paper was cited by the Examiner of the above-identified application, when rejecting claims 1, 3-4 and 13-15 under 35 USC § 103 in an Office Action dated January 24, 1996.

3. The disclosure set forth in the accompanying Wu et al. publication, as it pertains to certain embodiments of the present invention, is a disclosure by myself of subject matter that I (as one of the present co-inventors) am claiming in the above-identified application. In support of this contention, a review of the accompanying reference shows that Figure 2 thereof corresponds to Figure 1 of the present application, Figure 8 corresponds to Figure 2 of the present application, and Tables 2-3 thereof correspond to Tables 1-2 of the present application; thus showing that the accompanying publication corresponds exactly with the present application's disclosure, in at least some aspects.

4. The disclosure that I made in the accompanying Wu et al. publication, occurred within one year of the filing date of U.S. Application Serial No. 07/551,353 (i.e., July 12, 1990), upon which priority for the above-identified application is claimed under 35 USC 120.

5. The accompanying Wu et al. publication names Dr. Y-W. Wu and Dr. C. L. Chik as co-authors with myself, since they worked under my direction and control at the Developmental Endocrinology Branch, NICHD, NIH, in helping to devise certain laboratory studies that were subsequently reported in the accompanying Wu et al. publication (as well as in the above-identified application).

6. Even though Dr. Wu and Dr. Chik are co-authors, with myself, of the accompanying Wu et al. publication, they are not co-inventors of the subject matter that is disclosed and claimed in the above-identified application. This is because they did not take part in the conception of the invention that is being disclosed and claimed in the above-identified application. Again, Dr. Wu (who worked under my direction and control as a "visiting fellow") and Dr. Chik (who worked under my direction and control as a "staff fellow"), mainly helped in devising the assay methods that were used in the laboratory studies that are reported in the accompanying Wu et al. publication.

7. I do not know the current address of either Dr. Wu or Dr. Chik, since we no longer work together at the Developmental Endocrinology Branch of NICHD. However, to the best of my knowledge, I believe that Dr. Wu (a Chinese national) now resides in Bejing, China, having moved there in about 1990. Concerning Dr. Chik, to the best of my knowledge, she now resides in Alberta, Canada, and is employed at a Canadian university. I have no further knowledge concerning either Dr. Wu's or Dr. Chik's current address or whereabouts.

8. While Mary R. Flack and Marcus Reidenberg were not listed as co-authors on the accompanying Wu et al. publication, they are nonetheless co-inventors (with myself) of the subject matter that is disclosed and claimed in the above-identified application. This is because, Mary R. Flack, Marcus Reidenberg and myself were each involved in the conception of the invention that is disclosed and claimed in the above identified application. In this regard, Mary R. Flack and Marcus Reidenberg were not listed as co-authors on the accompanying Wu et al. publication, since they were not directly involved with the laboratory studies reported in the Wu et al. publication, even though they were involved with clinical studies relating to the present invention.

9. The sole co-inventors of the subject matter that is disclosed and claimed in the above-identified application are myself (Richard Knazek), Mary R. Flack and Marcus Reidenberg.

10. I hereby declare that all statements made herein on my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United

States Code and that such willful statements may jeopardize the validity of the application or any patents issued therefrom.

Date: June 25, 1996 By: Richard Knazek
Richard Knazek

CURRICULUM VITAE

NAME: Richard Allan Knazek

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DATE AND PLACE OF BIRTH: March 23, 1942, Cleveland, Ohio

CITIZENSHIP: United States

MARITAL STATUS: Married, two children

EDUCATION:

1958-1962 B.S. Chemical Engineering, Case Institute of Technology Cleveland, OH

1962-1963 M.S. Chemical Engineering, Lehigh University, Bethlehem, PA (Thesis: The Flooding capacities of Pulsed Liquid-liquid Extraction Columns)

1964-1965 Evening classes in Biology and Biochemistry, University of Delaware

1965-1969 M.D., Ohio State University College of Medicine, Columbus, OH

1969-1971 Internship and Junior Residency, Duke Hospital, Durham, NC

1973-1974 Clinical Associate, Medical Oncology, NCI/NIH, Bethesda, MD

1980-1981 Clinical Associate, Endocrinology Fellowship Program, Clinical Center/NIH, Bethesda, MD

BRIEF CHRONOLOGY OF EMPLOYMENT:

1960-1962 (Summers) Junior Engineer in computer programming, engineering design and engineering development at Standard Oil of Ohio

1962-1963 Part-time, research on thermo-physical properties of cryogenic compounds, at Air Products and Chemicals Co., Emmaus, PA

1963-1965 Full-time engineering research at the Experimental Station at E.I. duPont Co. in Wilmington, DE

1966 (Summer) Research on artificial kidney with Dr. Robert Sparks at Case Institute of Technology, Cleveland, OH

1968-1969 Hematology research in the laboratory of Dr. Charles Mengel, Ohio State University College of Medicine, Columbus, OH

1971-1973	Research Associate (Surgeon, PHS) Laboratory of Biochemistry, Tumor-Physiopathology Section, NCI/NIH, Bethesda, MD
1973-1974	Research Associate (Senior Surgeon, PHS) Laboratory of Pathophysiology Section, DCBD, NCI/NIH, Bethesda, MD
1974-1985	Senior Investigator (Medical Director, PHS), LPP, NCI/NIH, Bethesda, MD
1974-1992	Physician in part-time private practice, self-employed, Rockville, MD
1983-1985	Visiting Professor, Institute of Pathology, Lausanne, Switzerland
1986-1988	Deputy Branch Chief (Medical Director, PHS), Developmental Endocrinology Branch, NICHD/NIH, Bethesda, MD
1988-1990	President and Co-founder, Cellco Advanced Bioreactors, Inc. (Cellco, Inc.), Kensington, MD
1990-1994	Senior Vice President, Cellco, Inc., Germantown, MD
1994-	Clinical Associate Professor, Division of Endocrinology, Diabetes and Clinical Nutrition, The Oregon Health Sciences University School of Medicine, Portland, OR
1994-	Visiting Scientist, Clinical Gene Therapy Branch, National Center for Human Genome Research, NIH, Bethesda, MD

RESPONSIBILITIES AT CELLCO, INC.:

Co-founded company in 1987.

Obtained first- and second-round venture capital financing.

Developed and commercialized three artificial capillary culture devices to grow large quantities of cells *in vitro*.

Established commercial corporate alliances and academic research relationships.

Filed device and process patent applications.

Focused technology to produce of lymphocytes and hematopoietic progenitor cells for cellular therapy protocols for cancer, AIDS and genetic disorders.

Directed in-house research and development programs for tumor infiltrating lymphocyte (TIL) therapy of cancer, cytotoxic lymphocyte therapy of AIDS and retroviral transduction of genetically defective lymphocytes.

Coordinated Cellco programs with the physician-sponsored pre-clinical and clinical cellular therapy trials of multiple academic institutions.

HONORS AND OTHER SPECIAL SCIENTIFIC RECOGNITIONS:

1967	Landacre Society -"for outstanding student research" - Ohio State University College of Medicine
1968	Honorable Mention - SAMA-University of Texas Medical Branch Scientific Forum
1969	Phi Delta Epsilon Senior Award - Ohio State University College of Medicine
1974	Diplomate in Internal Medicine - Certified by the American Board of Internal Medicine
1975	The Inventors Award - National Institutes of Health
1976	"Ten Outstanding Young Men" in America - U.S. Jaycees Award
1982	Diplomate in Endocrinology and Metabolic Diseases - Certified by the American Board of Internal Medicine
1985	The Foreign Duty Service Ribbon - U. S. Public Health Service

SOCIETY MEMBERSHIPS:

Endocrine Society
American Association for Cancer Research
Society for Experimental Biology and Medicine

OFFICE SKILLS:

Word processing, Display Write 3, Word, Word Perfect.

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3. Knazek RA, Kohler PO and Gullino PM. Hormone production by cells grown in vitro on artificial capillaries. Exptl. Cell Res. 84:251-259, 1973.

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[CANCER RESEARCH 49, 3754–3758, July 15, 1989]

An *in Vitro* and *in Vivo* Study of Antitumor Effects of Gossypol on Human SW-13 Adrenocortical Carcinoma

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ABSTRACT

The present study investigated the *in vitro* and *in vivo* antitumor effects of gossypol on human SW-13 adrenocortical carcinoma cells. *In vitro* gossypol concentrations ≥0.5 μM reduced the growth rate of the SW-13 cells. Membrane microviscosity was determined by fluorescence polarization of diphenylhexatriene. The membranes of viable SW-13 cells exposed to gossypol became more rigid after a 1-day exposure to gossypol, the polarization constant, *P*, increasing from 0.229 to 0.352. Gossypol also increased the microviscosities of isolated mitochondrial and microsomal enriched membrane preparations. Tumor was also transplanted into nude mice by s.c. injection of SW-13 cells. A 1-week pretreatment period followed by daily administration of gossypol in which 30 mg gossypol/kg body weight/day was administered via orogastric tube delayed the onset of visible tumor in the subsequent weeks. Five weeks after transplantation, tumor prevalence rate was 95.8% in the control group and 54.5% in the gossypol-treated group. A second experiment, consisting of 12 weeks of gossypol treatment, reduced a preexisting 71% tumor prevalence to 54% while the tumor prevalence increased to 83% in the control group. This was accompanied by a 41.6% mortality in the control group *versus* 8.3% in the gossypol-treated group. These data suggest that gossypol may provide a beneficial effect in patients with adrenocortical carcinoma.

INTRODUCTION

Gossypol is a naturally occurring component of cottonseed oil (Fig. 1). It has been shown to be an effective antispermatogenic agent in humans and certain experimental animals, including rats, hamsters, and cynomolgus monkeys (1–6). Gossypol has been shown to impair spermatogenesis by a number of mechanisms. Several investigators have recently reported that gossypol is an uncoupler of oxidative phosphorylation (7), that it both stimulates and inhibits respiration (8), and that it also reduces ATP production (9, 10). Other actions of gossypol include its effects on the activity of many membrane-associated enzymes. Gossypol inhibits several enzymes in the electron transport chain, including lactic dehydrogenase X, NAD-isocitrate dehydrogenase, succinyl-CoA synthetase (11, 12), as well as adenylate cyclase (13), phospholipid sensitive calcium-dependent protein kinase (14), and ATPase activity (15). At the membrane level, it also affects the electrochemical properties of lipid membrane and the ordering of membrane lipid matrix (16, 17).

Since gossypol exhibits such a broad spectrum of activities, several investigators have examined gossypol as an antitumor agent: gossypol lengthened the survival of 10–12-week-old C57BL × DBA/2 F_1 (hereafter called $BD2F_1$) mice bearing mouse mammary adenocarcinoma 755 (18) and was effective against the TR-ST cells originating from a rat testicular tumor (19). Gossypol also lengthened the survival of NMRI mice preimplanted with Ehrlich ascites tumor cells (20). Excessive i.p. doses of gossypol given in the latter experiments may have resulted in weight loss and consequent death of the tumor-bearing mice.

We have previously observed that gossypol increased the microviscosity of both mitochondrial- and microsomal-enriched membranes of normal human adrenocortical cells while inhibiting the steroidogenic activity of several adrenocortical enzymes and altering the sensitivity of adrenal cells to adrenocorticotropic hormone.[4] These observations and the possible antitumor properties led to the hypothesis that there might be a direct and preferential action of gossypol on adrenocortical carcinoma. For this reason, the *in vitro* and *in vivo* effects of gossypol on the SW-13 human adrenocortical carcinoma cells were studied

MATERIALS AND METHODS

Dulbecco's minimal Eagle's medium, fetal calf serum, glutamine, penicillin, and streptomycin were purchased from Quality Biological, Inc. (Gaithersburg, MD). HBSS[5] and trypsin-EDTA were obtained from Gibco Laboratories (Grand Island, NY). 1,6-Diphenylhexatriene and tetrahydrofuran were from Aldrich Chemical Co., Inc. (Milwaukee, WI). Gossypol and gossypol acetic acid were gifts from the National Research Institute for Family Planning (Beijing, China). The established line of small cell human adrenocortical carcinoma (SW-13) was purchased from the American Type Culture Collection (Rockville, MD).

In Vitro Gossypol Treatment

Cell Proliferation. SW-13 cells were seeded in a 25-cm^2 tissue culture flask (Costar, Cambridge, MA) at densities of 1×10^4 cells/5 ml of Dulbecco's minimal Eagle's medium, supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, 100 units/ml penicillin, and 2 mM glutamine. The cells were grown in a humidified, 37°C incubator with a 5% CO_2/95% air atmosphere. A gossypol stock solution in absolute ethanol was added to the culture medium to yield final concentrations of 0, 0.5, 5, and 50 μM gossypol with a 0.1% final ethanol concentration. After 1, 2, 4, or 6 days of incubation, the culture medium, containing a few floating cells, was removed. Adherent cells were trypsinized (0.1% trypsin, w/v) and counted using a hemocytometer. Cell viability was determined by trypan blue exclusion.

Membrane Microviscosity Measurements. Cultures of the SW-13 cells were rinsed twice with HBSS (pH 7.2), scraped from the culture dishes, centrifuged at 150 × *g* for 10 min, and homogenized in 0.3 M sucrose in HBSS, using a Teflon-glass homogenizer. After centrifugation at 800 × *g* for 10 min, the supernatant was removed and centrifuged at 15,000 × *g* for 20 min, and then at 100,000 × *g* for 60 min. The 15,000 × *g* and the 100,000 × *g* membrane pellets which comprised crude mitochondrial and microsomal preparations, respectively, were homogenized and resuspended in HBSS. The 1,6-diphenylhexatriene was dissolved in tetrahydrofuran at a concentration of 2 mM before being dispersed in HBSS, to provide a final concentration of 2 μM which was then sparged with N_2 for 30 min to remove traces of

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[2] Supported by a fellowship from the Medical Research Council of Canada.

[3] To whom requests for reprints should be addressed, at Developmental Endocrinology Branch, NICHD, NIH, Building 10/Room 10N262, 9000 Rockville Pike, Bethesda, MD 20892.

[4] Y. W. Wu, C. L. Chik, B. D. Albertson, W. M. Linehan, and R. A. Knazek. Effect of gossypol on human and rat adrenal cell membranes, submitted for publication.

[5] The abbreviation used is: HBSS, Hanks' balanced salt solution.

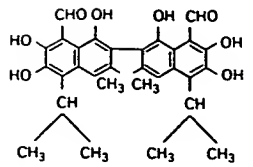


Fig. 1. Chemical structure of gossypol.

tetrahydrofuran before use. The membrane suspensions were diluted to concentrations of 50 μg protein/1.5 ml. Protein determination was made by a dye binding method using bovine serum albumin as the standard (21). These suspensions were then incubated with an equal volume of the HBSS-1,6-diphenylhexatriene dispersion for 1 h at room temperature. Each membrane sample was subsequently subjected to polarization analysis at 37°C using a Perkin-Elmer MPF-66 fluorescence spectrophotometer (Perkin-Elmer Corporation, Oak Brook, IL). The excitation wavelength was 366 nm, and the emission wavelength was 430 nm. Microviscosity is proportional to the value of fluorescence polarization (P) which was calculated according to the equation:

$$P = \frac{I_v - G \cdot I_H}{I_v + G \cdot I_H}$$

where I_v and I_H are the relative fluorescence intensities measured at an angle of 90 degrees to the incident beam with the emission polarizer in the vertical and horizontal positions, respectively, and $G = I_v/I_H$ measured with the excitation polarizer in the horizontal position.

In Vivo Gossypol Treatment

Nude Mice. Nude mice (Charles River, Kingston, NY) weighing 20–35 g were caged in a temperature-controlled (26–28°C), 12 h/12 h light/dark animal room. A microporous cage bonnet served as an effective protective barrier between the animal and the outside environment. In addition, the room was continuously purged with High Efficiency Particle Attenuator-filtered air. The cages, feeders, and water bottles were designed to make standard mouse chow and water readily available while minimizing the opportunity for the transfer of communicable pathogens.

Transplantation of SW-13 Cells. Forty-nine adult male nude mice weighing 20–24 g were divided into two groups of 24 for control and 25 for gossypol treatment. Gossypol acetic acid was suspended in 75% ethanol for 24 h, then evaporated in vacuum chamber with desiccant, and finally suspended in sterilized 0.25% carboxymethylcellulose carrier. The gossypol-treated group received 30 mg gossypol/kg body weight/day via an orogastric tube. Control mice were fed an equal volume of carrier. Body weights were measured weekly. At the end of the first week of gossypol treatment, 2×10^6 SW-13 cells were injected s.c. on the back of these mice, which continued to receive gossypol or carrier for 5 additional weeks. Tumor surface areas (length × width, cm^2) were measured daily. After 5 weeks, the animals were decapitated.

Another experiment was designed wherein 48 adult male nude mice weighing 25–35 g were injected s.c. with 2×10^6 SW-13 cells. One month later, the animals were divided into two groups of 24. There were 7 nude mice without visible tumors in each group. The gossypol-treated animals received 30 mg gossypol acetic acid/kg body weight/day whereas control animals were fed an equal volume of carrier. Body weights and tumor sizes (length × width, cm^2) were measured weekly. During the 12th week of treatment, 5 control animals died. Since it appeared unlikely that the remaining control animals would survive for another week, they were then sacrificed. Autopsies were performed on all animals including those that died during the study period. Internal organs were examined for the presence of gross tumor. Livers and tumors were rinsed with phosphate-buffered saline (pH 7.2) and homogenized in 0.3 M sucrose in phosphate-buffered saline, using a Teflon-glass homogenizer to prepare crude mitochondrial and microsomal membranes. The microviscosities of these membranes were then determined as described previously.

Statistical Analysis. Data are expressed as the mean ± SD unless otherwise indicated. Statistical comparisons were made using an unpaired Student's t test.

RESULTS

Proliferation of SW-13 Cells *in Vitro*

SW-13 cells were seeded in fresh medium. Upon exposure to gossypol at 0.5 μM they continued to proliferate at almost the same rate as control cells. Gossypol concentrations of 5 and 50 μM were inhibitory (Fig. 2).

Effect of Gossypol on the Microviscosity of SW-13 Cell Membranes *in Vitro*

Addition of gossypol to suspensions of either microsomal or mitochondrial membranes prepared from SW-13 cells resulted in a rapid, dose-dependent increase in their polarization constant, P. The values of P were then stable during the next 60 minutes (Figs. 3 and 4). Microsomes appear to be more sensitive to gossypol exposure than mitochondria, with 50% inhibitory concentrations of 0.56 μM for microsomes and 1.4 μM for mitochondria (Fig. 5).

After *in vitro* exposure of SW-13 cells to 50 μM gossypol for 2 days, the polarization constant, P, increased from 0.23 ± 0.01 on day 0 to 0.44 ± 0.04 on day 2 whereas P remained unchanged for the control cells incubated without gossypol (Fig. 6).

Effect of Gossypol on SW-13 Tumor Growth *in Vivo*

Study 1. One-Week Pretreatment with Gossypol. Gossypol administered for 6 weeks at a dosage of 30 mg/kg body weight/day by orogastric tube had no effect on body weights. Body weights of control animals at the end of 6 weeks were 24.5 ± 2.5 g compared to 24.6 ± 3.3 g for gossypol-treated animals. After a 1-week pretreatment period with either gossypol or carrier, all animals were inoculated with 2×10^6 SW-13 cells. The subsequent prevalence and average of tumor sizes are shown as a function of time in Table 1. The time required for 50% of the mice to develop tumors were 19 and 30 days for the control and gossypol groups, respectively. The total tumor burden within the control group increased over the 5 weeks subsequent to cell inoculation, reaching a value 4.5 times that of the gossypol-treated group (Fig. 7).

Study 2. Effect of Gossypol on SW-13 Tumor-Bearing Nude Mice. In this experiment, nude mice had been given s.c. injections of SW-13 adrenocortical carcinoma 1 month prior to initiation of the treatment with either gossypol or carrier.

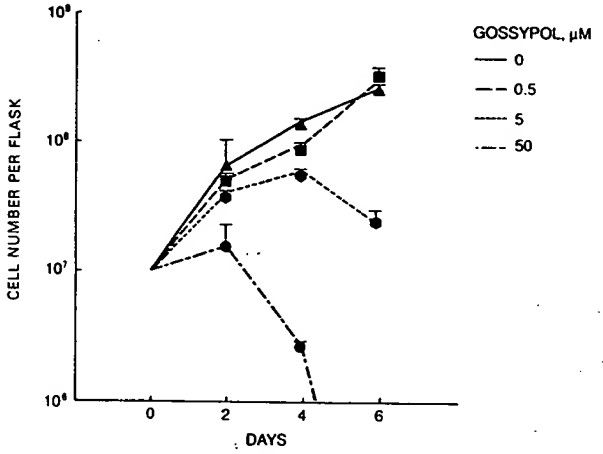
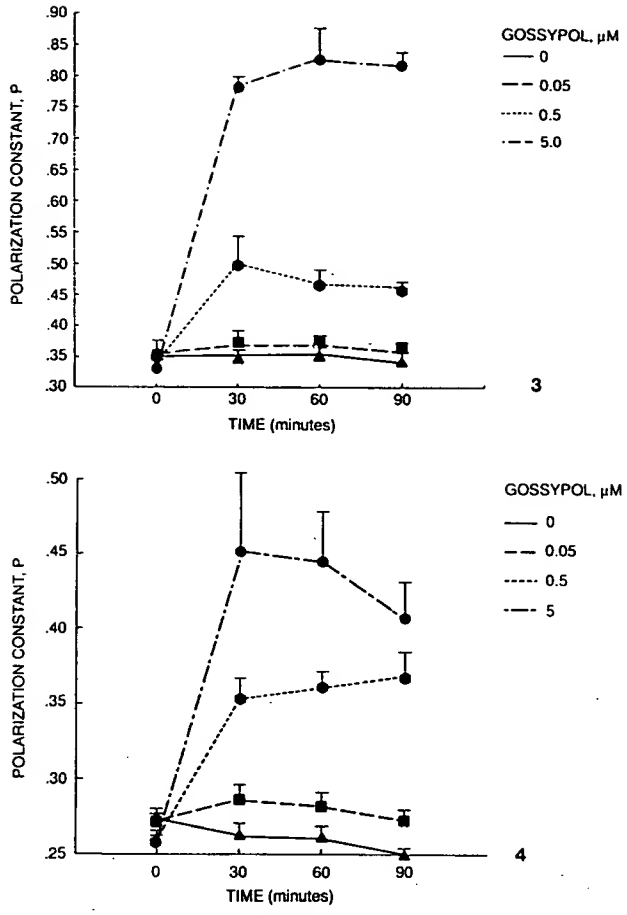


Fig. 2. Proliferation of SW-13 cells during a prolonged exposure to concentrations of 0, 0.5, 5, and 50 μM gossypol. The SW-13 cells were seeded (1×10^4 cells) into 25-cm^2 tissue culture flasks in Dulbecco's minimal Eagle's medium supplemented with fetal calf serum (10%), 100 μg/ml streptomycin, 100 units/ml penicillin, and 2 mM glutamine. Note that exposure to 5 and 50 μM gossypol inhibited cell proliferation.



Figs. 3 and 4. The fluorescence polarization constant, P, was determined in SW-13 human adrenocortical carcinoma cells membranes as a function of time after addition of various concentrations of gossypol *in vitro*. Since the value of P is known to be proportional to membrane microviscosity, the data indicate that exposure to gossypol results in an increase in the microviscosity of both the microsomal enriched (Fig. 3) and mitochondrial enriched (Fig. 4) preparations in a dose-related manner.

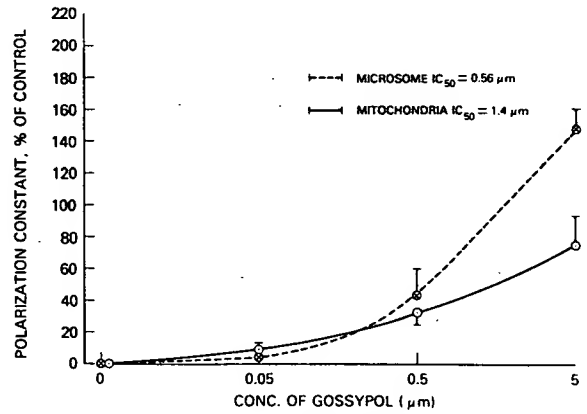


Fig. 5. Gossypol caused increase in the membrane microviscosity of the microsomal and mitochondrial membranes of SW-13 cells *in vitro*. The microsomes appeared to be more sensitive to gossypol exposure than mitochondria, a 50% inhibitory concentration (IC_{50}) of 0.56 μM for microsomes *versus* 1.4 μM for mitochondria.

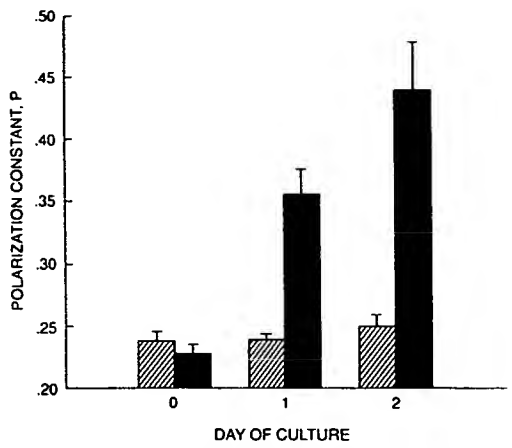


Fig. 6. Incubation of SW-13 cells with 50 μM gossypol caused an increase in the value of the fluorescence polarization constant, P, with time. Gossypol: ▨, 0; ■, 50 μM.

Table 1 *Effects of 1 week pretreatment followed by daily administration of gossypol on tumor prevalence and size*

	Control		Gossypol	
Weeks	Prevalence of tumor (%)	Av. tumor size (cm^2)	Prevalence of tumor (%)	Av. tumor size (cm^2)
1	12.5	0.08	0	0
2	20.8	0.10	0	0
3	66.7	0.30	33.3	0.08
4	83.3	0.39	34.7	0.09
5	95.8	0.41	54.5	0.11

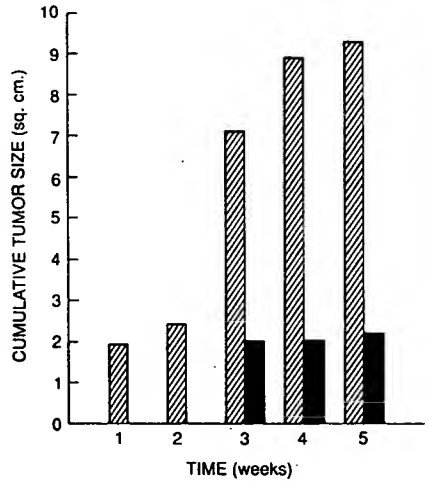


Fig. 7. Gossypol treatment caused a decrease in the cumulative tumor surface area. The tumor sizes (length × width, cm^2) were measured daily. Tumor-bearing mice received gossypol at a dose of 30 mg/kg/day. The data are presented on a weekly basis. Gossypol (mg/kg/day): ▨, 0; ■, 30.

During the subsequent 12 weeks of treatment, there were 10 deaths in the control group: 4 had apparent ascites, were jaundiced, and had large intraperitoneal tumors; 2 suffered from hind leg paralysis due to a tumor metastatic to the spinal column; 2 animals had small tumors but both showed significant weight loss; and 2 had demonstrated neither visible tumors nor an obvious cause of death. In contrast, only two deaths were observed in the gossypol-treated group, one of them having ascites while the other had no apparent tumor at autopsy. Each treated mouse in the group received a total dose of 81.9 mg gossypol during the 12-week period.

As in the previous study, 12 weeks of gossypol treatment had no significant effect on body weights. At the end of the study period, the body weights in both groups were 32.2 ± 3.8 and 30.9 ± 3.6 g for the control and gossypol-treated groups, respectively. After 12 weeks of treatment, the tumor prevalence had risen from 71 to 83% in the control group, while the gossypol-treated group exhibited a decrease in tumor prevalence from 71% to 54%. This was accompanied by the death of 41.6% of the controls and 8.3% of the gossypol-treated group (Table 2). The mean tumor sizes of the control and the gossypol-treated groups were shown as a function of duration of treat-

Table 2 *Effect of gossypol on tumor prevalence and mortality in mice having preexisting tumors*

Week	Control (%)		Gossypol (%)	
	Prevalence of tumor	Total deaths	Prevalence of tumor	Total deaths
0	71	0	72	0
1	75	0	63	0
2	83	0	50	0
3	83	0	54	0
4	83	0	50	0
5	83	0	58	0
6	83	0	58	0
7	83	8.3	58	0
8	83	8.3	58	0
9	83	12.5	54	0
10	83	16.7	54	0
11	83	20.8	54	0
12	83	41.6	54	8.3

Table 3 *Effect of gossypol on mean tumor size*

Week	Mean tumor size (cm^2) (mean ± SE)	
	Control	Gossypol
0	0.09 ± 0.02	0.08 ± 0.02
1	0.22 ± 0.05	0.07 ± 0.02
2	0.28 ± 0.06	0.11 ± 0.04[a]
3	0.35 ± 0.07	0.15 ± 0.05[a]
4	0.50 ± 0.11	0.20 ± 0.07[a]
5	0.66 ± 0.17	0.28 ± 0.08[a]
6	0.87 ± 0.22	0.32 ± 0.10[a]
7	0.97 ± 0.25 (n = 23)	0.38 ± 0.12[a]
8	1.16 ± 0.33 (n = 22)	0.45 ± 0.14[a]
9	1.07 ± 0.34 (n = 20)	0.50 ± 0.15[a]
10	1.14 ± 0.36 (n = 20)	0.59 ± 0.18[a]
11	1.39 ± 0.41 (n = 19)	0.68 ± 0.21[a]
12	0.96 ± 0.21 (n = 15)	0.81 ± 0.25 (n = 22)

[a] $P < 0.05$, control compared to gossypol treated group; $n = 24$ unless otherwise indicated.

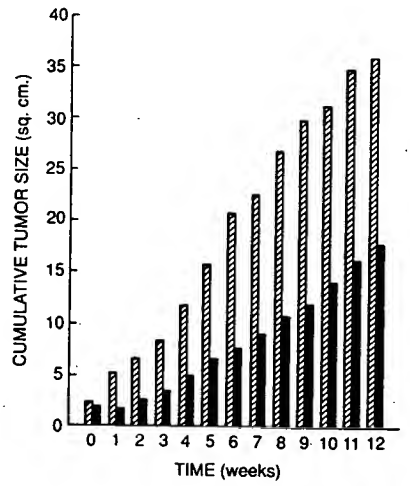


Fig. 8. Effect of gossypol on the cumulative tumor surface areas. The tumor size of each mouse was determined at weekly intervals and expressed as total tumor surface area present in each group. Gossypol (mg/kg/day): ▨, 0; ■, 30.

ment in Table 3. The slight decline in the mean tumor size observed towards the end of the study period was due to the fact that the majority of the control mice that died during the study had large tumors.

The total tumor burden of the two groups rose during the treatment period, the controls reaching a value twice that of the gossypol group at the 12th week (Fig. 8). The fluorescent polarization constants of the mitochondrial and microsomal preparations from the livers and tumors of these mice are shown in Table 4. No significant effect of gossypol upon their membrane microviscosity was noted in this *in vivo* study.

Table 4 *Polarization constant of liver and tumor membranes obtained from mice in Study 2*

No significant difference was noted between treated and control groups.

	Control		Gossypol	
	Liver	Tumor	Liver	Tumor
Mitochondria	0.25 ± 0.02	0.26 ± 0.03	0.26 ± 0.03	0.22 ± 0.04
Microsomes	0.26 ± 0.04	0.27 ± 0.04	0.26 ± 0.03	0.26 ± 0.03

DISCUSSION

Gossypol was shown to suppress the proliferation of SW-13 human adrenocortical carcinoma cells and increase the microviscosity of their membranes. A recent study utilizing artificial membranes indicate that gossypol both binds to phospholipid monolayers with apparent equilibrium dissociation constants ranging from 0.7 to 2 μM and diminishes the interfacial potential (16). The decreased rate of cell proliferation following gossypol treatment in the present study may, therefore, be a consequence of alterations of phospholipid membranes. Changes in the rigidity of such cell membranes may be a direct effect of gossypol within the membrane or may be secondary to compensatory changes in membrane structure or composition and consequent modification of membrane-associated enzymes.

Pretreatment with gossypol delayed the initial appearance of tumor by 13 days. Furthermore, tumors were detected in 95.8% of the control animals while 54.5% of the gossypol-pretreated group exhibited tumors. The cumulative tumor burden of the control group was approximately 4 times that of the gossypol-treated group. Such findings indicate that gossypol may effectively inhibit early tumor growth.

Established tumors were also shown to respond to gossypol treatment. Tumors eventually appeared in 83% of the control animals but decreased in prevalence from 71% initially to only 54% in the gossypol-treated group. In addition, 41% of the controls died *versus* only 8.3% of the treated group. Gossypol treatment, therefore, appears to lengthen the survival of SW-13 tumor-bearing male nude mice. Two previous studies have also indicated that gossypol doses of 25–100 μg/mouse/day or single injections of 0.5 mg/mouse can lengthen survival of NMRI mice bearing Ehrlich ascites tumor cells and 10–12-week-old $BD2F_1$ mice bearing mouse mammary adenocarcinoma 755. However, the gossypol treatment of these previous studies appear to be toxic (18, 20). One major difference between the present and previous studies was the route of administration of gossypol. In the other experiments, gossypol was injected i.p., which in itself may have resulted in higher toxicity. In the present experiment, gossypol was given into animals via an orogastric tube. A cumulative dose of 81.9 mg gossypol during the 12-week period had no effect on body weight and was accompanied by only one incidental death.

Although gossypol appeared to be safe and effective in delaying growth of SW-13 cells in nude mice, the mechanism of the antitumor action of gossypol remains to be determined. We have previously demonstrated that gossypol inhibits the function of normal human adrenocortical cells, perhaps by increasing the microviscosity of the microsomal and mitochondrial membranes thereby altering the functionality of the membrane-associated steroidogenic enzymes.[4] The apparent predilection of gossypol to accumulate in the adrenal gland *in vivo* (22) suggested further that this agent might be useful in suppressing the function and growth of adrenocortical carcinoma. The data presented in this paper support this hypothesis, at least with regard to the human adrenocortical carcinoma studied in the nude mouse host. In the *in vitro* study, the membrane micro-

viscosity of the tumor was increased by gossypol treatment, indicating that this generalized membrane effect may be the mechanism by which gossypol could conceivably exert its antitumor effects. However, chronic *in vivo* treatment with gossypol failed to show a significant effect on membrane microviscosity except for a marginal effect on the mitochondrial membranes of gossypol-treated tumors. However, other studies have shown that *in vivo* exposure to agents or environmental conditions that modify membrane microviscosity will result in compensatory changes in the membrane composition to maintain the normal microviscosity. Such may be the case in the present *in vivo* studies which contrast with the *in vitro* experiments in which a marked sensitivity of the membrane microviscosity to gossypol was demonstrated. Compositional studies of the membrane exposed *in vivo* remain to be performed.

The findings of the present study may be of clinical significance in the treatment of adrenocortical carcinoma. Gossypol caused significant regression of preexisting SW-13 tumors and a reduction in overall mortality. The appearance of transplanted SW-13 tumors was also delayed by gossypol pretreatment. These data suggest that gossypol may provide a beneficial effect in patients with adrenocortical carcinoma by decreasing the overall tumor burden and prolonging their duration of survival.

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